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(71) Applicant: SERAGEN INC. [US/US]; 97 South Street, Hopkinton, MA 01748 (US).

(72) Inventors: SVRLUGA, Richard, C. ; 1795 Beacon Street, Brookline, MA 02146 (US). WATERS, Cory, A. ; 12 Clark Road, Bedford, MA 01730 (US).

(74) Agent: FRENCH, Timothy, A.; Fish & Richardson, One Financial Center, Suite 2500, Boston, MA 02111-2658 (US).

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(57) Abstract

A hybrid molecule containing a hematopoietin receptor superfamily cytokine (or a fragment or analog thereof capable of binding to the high-affinity receptor for such cytokine) covalently bonded, at its amino terminal end, to a chemical entity which causes the hybrid molecule to be processed via a lower affinity receptor for such cytokine to a degree less than the degree of processing of the cytokine by the lower affinity receptor, provided that the chemical entity cannot be a fragment of diphtheria toxin which includes the translocation domain of that toxin.

* See back of page

HYBRID MOLECULES

Background of the Invention

The field of the invention is cytokine hybrid molecules.

The hematopoietin receptor superfamily (Idzerda et al., J. Exp. Med. 171:861-873, 1990) is a family of cytokine receptors, the extracellular domains of which exhibit a significant degree of amino acid homology with each other. These receptors, which include the Interleukin 2 (IL-2) p75 receptor, are located on the surfaces of hematopoietic cells, including both lymphoid cells and myeloid cells, and are capable of binding and mediating the cellular proliferative effects of such cytokines as IL-2, Interleukin 3 (IL-3), Interleukin 4 (IL-4), Interleukin 6 (IL-6), erythropoietin (EPO), and prolactin. The cytokines which bind to members of the hematopoietin receptor superfamily are herein collectively termed "hematopoietin receptor superfamily cytokines".

The ability of IL-2 to bind specifically to IL-2-receptor-bearing cells, such as allograft-activated human T-cells and certain leukemic lymphocytes, has inspired the construction of toxin-IL-2 hybrid molecules capable of targeting their toxic effect specifically on cells displaying these IL-2 receptors ("IL-2R") on their surfaces. Although some of these hybrid molecules have been created by chemical conjugation of IL-2 with a toxin (Greenfield et al., Science, pp. 238, 536 (1987), more recently, recombinant DNA methodologies have been applied to the genetic assembly of hybrids such as diphtheria toxin/IL-2 hybrid (U.S. Patent No. 4,675,382) and Pseudomonas exotoxin A/IL-2 hybrid (Lorberboum-Galski et al., Proc. Natl. Acad. Sci. USA 85:1922-1926, 1988), in which IL-2 replaces the endogenous cell-specific receptor binding domain of the toxin protein. Both

native diphtheria toxin and native Pseudomonas toxin kill cells by binding to certain receptors on the surface of a susceptible cell, passing through the cell membrane, and, once inside the cell, shutting down protein synthesis by inactivating a crucial protein synthesis factor. The mechanism of cell killing by recombinant toxin-IL-2 hybrids is the same, except that the toxin's own cell-binding domain is replaced with IL-2, so that the hybrid toxin binds to IL-2-receptor-bearing cells exclusively.

The nature of these IL-2-specific receptors has been the object of several studies (e.g., Robb et al., J.Exp.Med. 154:1455-1464, 1981; Tsudo et al., Proc. Natl. Acad. Sci. USA 83:9694-9698, 1986; and Teshigawara et al., J.Exp.Med. 165:223-234, 1987). Two separate IL-2-binding receptor molecules, a 55 kilodalton glycoprotein ("p55" or "Tac") and a 75 kilodalton glycoprotein ("p75"), have been reported on human lymphocytes; each of these binds IL-2 alone or together in a heterodimeric receptor complex (Yagita et al., Cancer Res. 49, in press, 1989). Where the IL-2-binding molecule is p55 alone, IL-2 binds with relatively low affinity ($K_d = 10^{-8}M$); p75 alone binds IL-2 with an intermediate affinity ($K_d = 10^{-9}M$); and the heterodimeric form ("p75 + p55") binds with relatively high affinity to IL-2 ($K_d = 10^{-11}M$). The N-terminal amino acid residues of IL-2, particularly Asp_{20}, are essential for binding of IL-2 to the p75 receptor molecule (Collins et al., Proc. Natl. Acad. Sci. USA 85:7709-7713, 1988).

It has been shown that picomolar quantities of a diphtheria toxin/IL-2 recombinant hybrid are sufficient to inhibit protein synthesis by 50% in cells bearing the heterodimeric p75+p55 ("high affinity") type of IL-2 receptor, while cells which express only the p55 ("lowest affinity") or the p75 ("intermediate affinity") type of receptor are resistant to the protein-synthesis-

inhibiting effect of the same diphtheria toxin/IL-2 hybrid (Waters et al., Eur. J. Immunology 20:785-791, 1990).

In contrast, Pseudomonas exotoxin A/IL-2 recombinant hybrid has been shown to be internalized by the low and intermediate affinity receptor subunits as well as by the heterodimeric high affinity receptor (Lorberbaum-Galski et al., J. Biol. Chem. 263:18650-18656, 1988). In these constructions, the Pseudomonas exotoxin is linked to IL-2 through IL-2's carboxy-terminus.

Recently, all of the hematopoietin receptor superfamily cytokines have been demonstrated to have multiple receptors composed of distinct subunits of different binding affinities (cf. Itoh et al., Science 247:324-327, 1990; and Sawyer, Clinical Biotechnology 2:77-85, 1990).

Summary of the Invention

In general, the invention features a hybrid molecule containing a hematopoietin receptor superfamily cytokine (or a fragment or analog thereof capable of binding to the high-affinity receptor for such cytokine) covalently bonded, at its amino terminal end, to a chemical entity which causes the hybrid molecule to be processed via a lower affinity receptor for such cytokine to a degree less than the degree of processing of the cytokine by the lower affinity receptor, provided that the chemical entity cannot be a fragment of diphtheria toxin which includes the translocation domain of that toxin, as such domain is described in John R. Murphy U.S. Patent No. 4,675,382, hereby incorporated by reference. A "lower affinity receptor" for a given cytokine is any receptor capable of processing that cytokine, other than the high-affinity receptor for that cytokine. "Processing" is a term which is used to mean the binding

of a cytokine by one of that cytokine's receptors and internalization of the receptor-bound cytokine into an endocytic vesicle or into the cell. (For those hybrid molecules of the invention which are not internalized, the term "processing" means simply the binding of the cytokine by one of that cytokine's receptors.) Thus, a cytokine molecule is said to be "processed via a lower affinity receptor for such cytokine" when the cytokine molecule has bound to a low or intermediate-affinity receptor for that cytokine and has also been internalized by the cell (for example, in an endocytic vesicle) as a ligand/ receptor complex. The degree of such processing of a hybrid molecule of the invention can be quantitatively compared to the degree of processing of a naturally-occurring cytokine molecule by, for example, measuring the amount of each of such molecule which is internalized by cells bearing one type of receptor. The ability of a hybrid molecule to distinguish between high and lower affinity receptors is determined by comparing the amount of the molecule internalized by cells bearing only high-affinity receptors to the amount internalized by cells bearing only the lower affinity receptors: those hybrids which are capable of distinguishing between receptor types to a significant degree are useful for the purposes cited herein.

A "fragment" of a cytokine molecule is a polypeptide having an amino acid sequence exactly corresponding to some portion of the naturally-occurring molecule that is less than all of that naturally-occurring molecule, and can be generated, for example, by proteolytic digestion of the naturally-occurring molecule, by chemical synthesis, or by recombinant DNA techniques. An "analog" of a cytokine molecule is a polypeptide which differs from the naturally-occurring cytokine in that it has substitutions, additions, and/or

deletions of one or more amino acids at one or more locations: for example, an analog of a given cytokine would include those polypeptides which contain a contiguous segment at least half as long as the naturally-occurring cytokine and which has at least 80% sequence homology with some portion of the naturally-occurring molecule. The amino acid substitutions may be either conservative or non-conservative, and may be designed, for example, to remove proteolytically sensitive sites from the polypeptide. [By conservative is meant that the substituted amino acyl residue is chemically similar (e.g., acidic, basic, hydrophobic, aromatic) to the residue for which it is substituted: for example, substitution of a valine for a leucine.] Such analogs may be conveniently obtained by recombinant DNA techniques, or by any other method known to those in the field. Once generated, any such fragments or analogs can be tested for their ability to be processed via the high-affinity and the lower affinity receptors of the cytokine from which they are derived. Those which behave comparably to the naturally-occurring cytokine can be incorporated into the hybrid molecules of the invention.

In preferred embodiments,

the cytokine is selected from the group consisting of IL-2, IL-3, IL-4, IL-6, EPO, and prolactin;

the chemical entity interferes with the binding by the cytokine portion of the hybrid molecule to the lower-affinity receptor;

the chemical entity is an X-ray-opaque moiety, a fluorescent moiety, a radioactive moiety, a polypeptide (herein defined as two or more amino acids linked by peptide bonds) such as an antibody molecule, ricin, streptavidin, gelanin, or ferritin, or an enzymatically-active fragment or analog of such a polypeptide;

the covalent bond is a peptide bond; and

the hybrid molecule is capable of affecting the rate of proliferation of cells bearing high-affinity receptors.

The hybrid molecule of the invention may be used in a method for selectively binding a moiety to a cell having a high-affinity receptor for the cytokine of interest, by exposing the cells to hybrid molecule which contains the moiety covalently bonded to the amino terminus of cytokine (or a fragment or analog thereof capable of binding to the high-affinity receptor). In preferred embodiments, the moiety includes a label and the method is used for imaging high-affinity receptors or cells bearing high-affinity receptors; or the moiety is a molecule complexed with iron and the method is used for separating cells having a relatively high number of the high-affinity receptors from other cells having a lower number of such high-affinity receptors; or the hybrid molecule is capable of decreasing the rate of proliferation of cells to which it binds, and the method is used for therapeutic treatment of a condition characterized by overproduction of cells having high-affinity receptors.

The hybrid molecule of the invention can also be used in a method for selectively isolating cells having high-affinity receptors for the cytokine of interest, out of a population of cells some of which lack such high-affinity receptors, which method involves (1) immobilizing the hybrid molecule, (2) causing the population of cells to contact the immobilized hybrid molecules under conditions permitting binding of high-affinity receptors to the immobilized hybrid molecules, and (3) separating unbound cells from bound cells. This method may be used to remove, from bodily fluids or tissues, cells having such high-affinity receptors; alternatively, the method includes the additional step of

eluting the bound cells from the immobilized hybrid molecules, and is used for isolating, from bodily fluids or tissues, cells bearing high-affinity receptors.

The hybrid molecule in which the chemical entity is a polypeptide may be made, for example, by providing a recombinant DNA molecule containing a DNA sequence encoding that polypeptide fused to a DNA sequence encoding the cytokine of interest (or a high-affinity-receptor-binding fragment or analog thereof), introducing the recombinant DNA molecule into an appropriate expression system, and expressing the recombinant DNA molecule.

The streptavidin-containing hybrid molecule of the invention may be used in a method for imaging high-affinity receptors specific for the cytokine of interest, or, in a population of cells, a cell bearing such high-affinity receptors, by (a) exposing the high-affinity receptors to an amount of the hybrid molecule sufficient substantially to maximize the difference between (1) the amount of the hybrid molecule which binds to the high-affinity receptors and (2) the amount which binds to all lower-affinity receptors on the population of cells; (b) exposing the receptor-bound hybrid molecules to a labelled probe covalently linked to biotin; and (c) detecting the labelled probe which binds to the receptor-bound hybrid molecules.

The invention takes advantage of the ability of each of the hematopoietin receptor superfamily cytokines, when linked at its N-terminus to a chemical entity, to be processed via its lower-affinity receptors to a lesser degree than is the cytokine itself, resulting in enhanced discrimination between high- and lower-affinity receptors. This enhanced discrimination between receptor types by the hybrid molecule of the invention permits selective labelling, isolation, removal, or

killing of cells bearing the high-affinity receptor, to the virtual exclusion of cells lacking this receptor, and thus presents an important tool for the study, diagnosis, and therapeutic treatment of various conditions in which myeloid or lymphoid cells are implicated as critical elements. For example, a hybrid molecule of the invention having IL-2 as the cytokine portion would target the high-affinity receptors on activated T cells, and so would be useful for applications involving transplant rejection, autoimmune disease, and certain lymphatic cancers.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof and from the claims.

Description of the Preferred Embodiments

The drawings will first briefly be described.

Drawings

Fig. 1 is a representation of the DNA coding sequence, and the corresponding amino acid sequence, for the IL-2 gene portion of plasmid pDW15, following SphI digestion of the plasmid.

Fig. 2 is a diagram illustrating the stepwise construction of plasmid PSI130 bearing the CRM197/IL-2 recombinant gene.

Fig. 3(a) is an illustration of a recombinant DNA plasmid intermediate in the construction of a ricin A/IL-2 hybrid gene.

Fig. 3(b) is an illustration of a recombinant DNA vector bearing the ricin A/IL-2 hybrid gene and suitable for expression in E.coli.

Fig. 4(a) is an illustration of a recombinant DNA plasmid intermediate in the construction of a streptavidin/IL-2 hybrid gene.

Fig. 4(b) is an illustration of a recombinant DNA vector bearing the streptavidin/IL-2 hybrid gene and suitable for expression in E.coli.

Discrimination Among IL-2 Receptors By IL-2 Hybrid And Native IL-2.

The invention is based upon the observation that attachment of a chemical entity to the N-terminal region of IL-2 interferes with processing of the IL-2 portion of the hybrid via intermediate-affinity (p75) receptors on T-cells, possibly due to structural constraints imposed on the chemical entity portion of the hybrid by the p75 receptor. Using an assay comparing (1) the molar concentration of a diphtheria toxin/ IL-2 hybrid molecule necessary to displace 50% of ^{125}iodine-labelled-IL-2 from binding sites on three cell lines, each of which displays only one of the three types of IL-2 receptors, with (2) the molar concentration of IL-2 necessary to effect the same displacement, it was found that, on a cell line displaying only the p55 receptor molecule, 10-fold more IL-2 hybrid was required to displace the labelled IL-2, while for cells displaying only the p75 receptor, the hybrid molecule had to be present at a concentration 100-fold higher than the concentration of IL-2 necessary to effect 50% displacement of labelled IL-2. Cells displaying the high-affinity (p75+p55) receptor required approximately a 100-fold higher concentration of IL-2 hybrid than of IL-2 to effect 50% displacement. Thus, (a) the affinity of hybrid-IL-2 for the p55 receptor is within an order of magnitude of the affinity of IL-2 for that receptor, (b) the affinity of hybrid-IL-2 for the p75 receptor is about 1% of the affinity of IL-2 for that receptor, and (c) the affinity of hybrid-IL-2 for the high-affinity (p75+p55) receptor is about 1% of the affinity of IL-2 for that receptor.

While these binding data suggest a slight binding differential between IL-2 and IL-2 hybrid for cells bearing only high-affinity receptors and those bearing only the lower-affinity p75 receptors, the net result of attaching the chemical entity to the N-terminus of IL-2 is a 300-1000-fold alteration in processing of the hybrid ligand, such that processing, in this instance measured as an IC_{50} for half-maximal inhibition of protein synthesis, is 300-1000-fold more efficient for the high-affinity-receptor-bearing cells than for lower-affinity-receptor-bearing cells.

The experimental results given above indicate that the major alteration in the ligand/receptor interaction caused by attaching a chemical entity to the amino terminus of IL-2 takes place with respect to the p75 receptor. The fact that the p75 receptor shares a high sequence homology with certain subunits of the receptors for all of the hemopoietin receptor superfamily cytokines suggests that placing a chemical entity such as a polypeptide at the N-terminus of any of these cytokines will inhibit, probably by steric hindrance, the processing of (i.e., the binding to and internalization of) the hybrid molecule by the subunit of the cytokine's receptor which is homologous with the p75 subunit of the IL-2 receptor, permitting the hybrid molecules of the invention to discriminate between their high-affinity receptors and their lower-affinity receptors to a significantly greater degree than does either the native cytokine or a cytokine hybrid constructed without introducing such steric hindrance near the binding domain analogous to the IL-2 p75 binding domain. This enhancement of the level of discrimination can be exploited in a number of ways, some of which are described below.

Example 1. Construction and expression of CRM197/IL-2 recombinant fused gene.

The IL-2 gene used for these fusions (Fig. 1) was obtained from plasmid pDW15 (Fig. 2) (Williams et al., Nucleic Acids Res. 16:10453-10467, 1988) containing a synthetic form of the IL-2 gene which, when cloned into E.coli JM101, expresses IL-2 protein at a rate about 16 times that of the native cDNA sequence cloned into the same strain of E.coli.

Standard DNA cloning techniques were employed. Plasmids were introduced into E.coli by $CaCl_2$ transformation, isolated by the alkaline lysis procedure, and purified by CsCl density gradient centrifugation (Maniatis et al., Molecular Cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982). The genetic fusion was made at the SphI site of pDW15 so that the IL-2 domain of the fused gene would encode 133 amino acids of IL-2, plus one additional amino acid on its amino terminus encoded by the Sph site (Fig. 1).

The construction methodology is illustrated in Fig. 2. Plasmid pABM6508, containing a gene coding for the N-terminal 485 amino acids of diphtheria toxin joined to α-melanocyte-stimulating hormone (Bishai et al., J. Bacteriol. 169:5140-5151, 1987), was digested completely with HindIII and partially with SphI; a 6kB SphI - HindIII vector fragment was gel-purified and ligated to the 0.5 kB SphI - HindIII IL-2-gene-containing fragment from pDW15, resulting in a plasmid designated pABI6508. A 5.7 kB vector fragment resulting from AccI and XmnI digestion of pABI6508 was gel-purified and ligated to a 0.8kB AccI - XmnI fragment of plasmid pß197 (Bishai et al.), yielding a plasmid that is referred to as pSI130. pß197 carries the gene for CRM197, which is a full-length (535

amino acids) $Gly_{52} \rightarrow Glu_{52}$ missense mutant form of diphtheria toxin that is devoid of ADP-ribosyl transferase activity and thus is nontoxic (Uchida et al., J. Biol. Chem. 248:3838-3844, 1973). The missense mutation occurs within the 0.8kB AccI - XmnI fragment of pß197, and so appears in pSI130 as well.

Expression of pSI130 in E.coli was induced as described by Bishai (J.Bacteriol. 1987); the CRM197/IL-2 gene product was purified using affinity chromatography (Williams et al. 1988) followed by HPLC size exclusion chromatography. (Methods in Enzymology 91: 137-190; 1983)

Example 2. Biological assay for IL-2 receptor discrimination.

Fifty micrograms of recombinant human IL (rIL-2) was enzymatically iodinated with enzymobeads (Bio-Rad Laboratories, Richmond, CA) in accordance with the manufacturer's instructions. One millicurie $Na^{125}I$ (DuPont-NEN, Boston, MA) was used in the reaction; this represented a limiting concentration of iodine; one atom of iodine to eight molecules of rIL-2. The reaction was allowed to proceed for five minutes at room temperature, and was quenched by the addition of NaN_3 and NaI. Fetal calf serum (FCS; Hyclone Laboratories, Logan, UT) was added to a final concentration of 10% and the mixture chromatographed through one milliliter of Sephadex G-10 which had been equilibrated with RPMI 1640 medium supplemented with 25 mM HEPES, (pH 7.4) (GIBCO, Grand Island, NY), 2 mM glutamine (GIBCO), 100 units/ml penicillin, 100 µg/ml streptomycin (GIBCO), and 10% FCS (Hyclone).

The radiolabeled IL-2 binding assay was performed essentially as described by Smith and co-workers (Robb et al.; Teshigawara et al.). Cells were harvested and

washed three times with RPMI 1640 medium containing 10% FCS. To determine total IL-2 binding, 5 x 10^5 cells were exposed to a known concentration of ^{125}I-rIL-2 (generally 1 x 10^{-9} M) for 30 minutes at 37°C in RPMI 1640 medium supplemented with 25 mM HEPES (pH 7.4), (GIBCO, Grand Island, NY), 2 mM glutamine (GIBCO), 100 units/ml penicillin and 100 µg/ml streptomycin (GIBCO), and 10% FCS (Hyclone)(total volume 150 µl). To determine the amount of rIL-2 or PT/IL-2 required to displace ^{125}I-rIL-2 from its receptor, increasing concentrations of unlabeled rIL-2 or PT/IL-2 (0.5- to 2000-fold molar excess) were added to each tube in the presence of 1 x 10^{-9} M ^{125}I-rIL-2 and the percentage of total binding at each concentration of cold competitor determined. The binding reaction was terminated by microcentrifuging (Beckman Instruments, Irvine, CA) the cells through an oil mixture of 80% 550 phenyl fluid (Dexter Hysol, Olean, NY) and 20% paraffin oil (Sigma, St. Louis, MO)(final density 1.03 gm/ml) in 400 microliter microcentrifuge tubes (Beckman Instruments). The cell pellet was cut from the microcentrifuge tube leaving the oil and medium containing free ligand in the tube. Cell pellets representing bound radioactivity and the oil/medium supernatants representing free ligand were counted in a Beckman gamma counter, and the percentage of the total radioactivity that pelleted with the cells was calculated. All assays were performed in triplicate.

Example 3. Construction and expression of Ricin A/IL-2 recombinant fused gene, and use of the resultant hybrid protein.

Standard procedures for DNA cloning, cell transformation and plasmid isolation (as described, for example, by Maniatis et al.), could be employed to carry out the following construction:

Ricin cDNA is prepared as described by Halling et al. (Nucleic Acids Res. 13:8019-8033, 1985) or Lamb et al. (Eur. J. Biochem. 148:265-270, 1985). Following digestion with BamHI and HaeIII, an 856-bp BamHI - HaeIII fragment of ricin cDNA (encoding the 5' end of the ricin gene, the signal sequence and ricin amino acids 1 through 272) is isolated by agarose gel electrophoresis.

An unphosphorylated synthetic DNA oligomer, comprising

5' AAGCGTCGGCATG 3'
3' TTCGCAGCC 5'

and coding for a protease-sensitive region (Lys Arg) and one-half of a SphI site (CATG), is synthesized by standard ß-cyanoethyl phosphoramidite chemistry on a Milligen 7500 DAN synthesizer according to manufacturer's instructions. The synthetic oligonucleotides are purified by gel electrophoresis, complementary strands are annealed and ligated to the HaeIII blunt end of the ricin gene fragment. The sequence of this oligomer was selected (1) to allow fusion of the ricin gene fragment to an IL-2 gene having the matching half of a SphI site, and (2) to allow release of enzymatically-active ricin A peptide from the ricin A/IL-2 polypeptide by proteolytic cleavage at the added protease-sensitive region upon internalization of the toxin molecule by a cell.

In order to insert a second half-SphI site at the appropriate spot upstream of the ricin coding sequence, the above construct is next digested with FnuDII (or ThaI) and the ~740 bp FnuDII - (HaeIII/SphI) fragment that results is isolated and

linked (at the FnuDII blunt end) to the following unphosphorylated synthetic oligonucleotide:

5' CC ATC GCT ATA TTC CCC AAA CAA TAC CCA ATT ATA-

3' GTACGG TAC CGA TAT AAG GGG TTT GTT ATG GGT TAA TAT-

encoding: **fMet Ala Ile Phe Pro Lys Gln Tyr Pro Ile Ile-**

(cont.): -AAC TTT ACC ACA GCG GGT GCC ACT GTG CAA AGC TAC ACA AAC-

-TTG AAA TGG TGT CGC CCA CGG TGA CAC GTT TCG ATG TGT TTG-

-Asn Phe Thr Thr Ala Gly Ala Thr Val Gln Ser Tyr Thr Asn-

(cont.): -TTT ATC AGA GCT GTT CG 3'

-AAA TAG TCT CGA CAA GC 5'

-Phe Ile Arg Ala Val Arg

This DNA sequence, which has a half of an SphI site at each end, can then be ligated into SphI partially-digested (linearized) plasmid pDW15 as described in Example 1. This plasmid is then digested with NcoI and HindIII, yielding a 1.3 kb DNA fragment coding for all of the following: mature ricin A, part of the ricin A linker, the synthetic protease-sensitive site, and IL-2.

This 1.3 kb NcoI - HindIII fragment is isolated and cloned onto NcoI - HindIII digested pKK233-2 vector (Fig. 3(b)) (Pharmacia; Piscataway, NJ; Armann et al., Gene 40:183-190, 1985) for expression in E.coli. Expression of the ricin A/IL-2 protein is induced by isopropyl-ß-D-thiogalactoside (IPTG). The protein may be purified by immunoaffinity chromatography followed by HPLC size exclusion chromatography.

Example 4. Construction and expression of streptavidin/IL-2 recombinant fused gene, and use of the resultant hybrid protein in imaging high-affinity receptors.

Standard procedures for DNA cloning, cell transformation, and plasmid isolation would be employed to carry out the following construction:

The streptavidin gene is isolated from Streptomyces avidinii as described by Argarana et al., Nucleic Acids Res. 14:1871-1882, 1986. Following NaeI digestion of the gene, a ~433 bp fragment is isolated and further digested with TaqI. A ~414 bp NaeI - TaqI fragment is thereupon isolated and its 5' NaeI blunt end is ligated with the following synthetic DNA oligomer (synthesized on a Milligen 7500 DNA synthesizer according to manufacturer instructions):

```
5'      CC ATG GAC CCC TCC AAG GAC TCG AAG GCC CAG
GTC-
3'  GTACGG TAC CTG GGG AGG TTC CTG AGC TTC CGG GTC
CAG-
encoding: fMet Asp Pro Ser Lys Asp Ser Lys Ala Gln
Val-
 (cont.): -TCG GCC GCC GAG GCC          3'
          -AGC CGG-CGG CTC CGG          5'
          -Ser Ala Ala Glu Ala
```

The following synthetic ligomer is ligated onto the (3') TaqI end of the fragment:

5' C GAC GCG GCG AAG AAG GCC GGC GTC AAC AAC GGC-

3' TG CGC CGC TTC TTC CGG CCG CAG TTG TTG CCG-

encoding: **Asp Ala Ala Lys Lys Ala Gly Val Asn Asn Gly-**

(cont.): -AAC CCG CTC GAC GCC GTT CAG CAG CAT G 3'

-TTG GGC GAG CTG CGG CAA GTC GTC 5'

-Asn Pro Leu Asp Ala Val Gln Gln His

The synthetic sequences so added replace all of the streptavidin coding sequences removed in the course of the NaeI and TaqI digestions, and add one half of a SphI site at each end of the fragment. This fragment can then be ligated into SphI partially-digested linearized pDW15 (see Example 1) and transformed into E.coli. The appropriate product of the ligation, which can be identified by plasmid restriction site mapping of DNA obtained from single colonies resulting from the transformation, is illustrated in Fig. 4(a). Following NcoI - HindIII digestion of this plasmid, a 1.0 kb NcoI -HindIII fragment containing the entire coding region for the streptavidin/IL-2 fusion protein is isolated and cloned onto NcoI - HindIII-digested pKK233-2 vector for expression in E.coli. The final form of this streptavidin/IL-2-encoding vector is illustrated in Fig. 4(b). E.coli calls are transformed and the streptavidin/IL-2 polypeptide is isolated and purified by immunoaffinity chromatography followed by HPLC size exclusion chromatography, and used, for example, to assay for

the presence of high-affinity IL-2 receptors on lymphoma cells.

Frozen or paraffin sections of a human lymphoma biopsy are prepared on microscope slides by standard techniques. Prior to staining, frozen sections are first fixed with acetone or another suitable fixative, then washed with a buffer such as .1m Tris, pH 7.2. Unlabeled biotin and/or free fluorochrome is then added to saturate all available nonspecific binding sites. After removal of unbound biotin by rinsing in buffer, the slides are treated with streptavidin/IL-2 hybrid protein and then rinsed in buffer to remove unbound streptavidin/IL-2. The slides are then treated with a solution containing a fluorochrome-biotin conjugate. The slides are again rinsed in buffer to remove unbound labeled biotin, and observed under a fluorescence microscope illuminated at an appropriate wavelength of light. The presence of a high proportion of fluorochrome-labeled cells in a biopsy of a lymphoma would be an indicator of that tumor's potential susceptibility to chemotherapeutic treatment with diphtheria toxin/IL-2 hybrid or another IL-2 N-terminus-linked toxic hybrid.

Other Embodiments

Other embodiments are within the following claims. For example, the cytokine portion of the hybrid molecule could be contributed by any of the hematopoietin receptor superfamily cytokines, or fragments or analogs thereof (prepared by standard techniques). The chemical entity could be ferritin, for use in separating cells bearing high affinity cytokine receptors from those which do not; an inert moiety, the function of which is simply to enhance the cytokine's discrimination between receptor types; or

an antigen, the function of which is to induce a host-mediated immunological attack on the host's own high-affinity-receptor-bearing cells. Alternatively, the chemical entity could be an enzyme, a carbohydrate, a lipid, a synthetic polymer, a viral particle, or an inorganic molecule. The linkage between the cytokine and the chemical entity can be a peptide bond or any other type of covalent bond. By using a chemical entity each molecule of which is capable of being linked to the N-termini of multiple cytokine molecules, a multivalent hybrid would be created which could bind simultaneously to two or more high-affinity-receptor-bearing cells, causing the cells to agglutinate and thus be cleared from a mixed lymphocyte suspension. This procedure would be useful as an assay or as a therapeutic technique.

The hybrid protein could be synthesized by utilizing recombinant DNA techniques, beginning with either a synthetic DNA sequence or a cloned natural gene, or could be assembled by chemically conjugating a chemical entity with the cytokine. After synthesis, the hybrid may be modified enzymatically or chemically (as by the addition of fluorescent or radioactive label). The polypeptide portion of any hybrid could be synthesized in a system incorporating one or more radioactive amino acids, so that the resulting hybrid protein is intrinsically labelled.

Any fragment or analog of the cytokine of interest which is capable of binding to the high-affinity receptor of that cytokine could substitute for the intact cytokine. An IL-2-containing hybrid could be used as a drug delivery device to deliver a lethal dosage of, for example, a radioactive isotope or a toxin such as ricin or gelanin specifically to high-affinity-receptor-bearing T-cells, which are

implicated in certain cancers of the lymphatic system, and which play a crucial role in cases of transplant rejection. Alternatively, the hybrid could be used to image high-affinity receptors, either *in vivo* or *in vitro*, by selecting an appropriate chemical entity to attach to the cytokine portion of the hybrid. Examples would include the treatment of a frozen section of lymphoma tissue with an IL-2 hybrid containing a radioactive isotope such as ^{125}Iodine, coupled with autoradiographic analysis of the labeled tissue, and administration of an IL-2 hybrid containing an X-ray-opaque entity to a transplant patient, coupled with a CAT-scan of the transplanted organ to monitor transplant rejection. Attaching a hybrid of the invention to an appropriate solid support material would create an efficient and reusable means of separating high-affinity-receptor-bearing T-cells from cells lacking such receptors, permitting the collection of either fraction of cells for therapeutic or other use.

What is claimed is:

Claims

1. A hybrid molecule comprising a hematopoietin receptor superfamily cytokine, or a fragment or analog of said cytokine capable of binding to said cytokine's high affinity receptor, covalently bonded, at its amino terminal end, to a chemical entity which causes said hybrid molecule to be processed via a lower affinity receptor of said cytokine to a lesser degree than is said cytokine, provided that said chemical entity does not comprise the translocation domain of diphtheria toxin.

2. The hybrid molecule of claim 1, wherein said cytokine is selected from the group consisting of IL-2, IL-3, IL-4, IL-6, EPO, and prolactin.

3. The hybrid molecule of claim 1, wherein said chemical entity sterically hinders the binding of the cytokine portion of said hybrid molecule to said lower-affinity receptor, compared to the binding of said cytokine to said lower-affinity receptor.

4. The hybrid molecule of claim 1, wherein said chemical entity comprises a polypeptide.

5. The hybrid molecule of claim 4, wherein said covalent bond is a peptide bond.

6. The hybrid molecule of claim 4, wherein said polypeptide comprises an antibody molecule.

7. The hybrid molecule of claim 4, wherein said polypeptide comprises ricin or a fragment or analog thereof.

8. The hybrid molecule of claim 7, wherein said polypeptide comprises an enzymatically-active fragment or analog of ricin.

9. The hybrid molecule of claim 4, wherein said polypeptide comprises streptavidin or a fragment or analog thereof.

10. The hybrid molecule of claim 4, wherein said polypeptide comprises ferritin.

11. The hybrid molecule of claim 1, wherein said chemical entity comprises an X-ray-opaque moiety.

12. The hybrid molecule of claim 1, wherein said chemical entity comprises a fluorescent moiety.

13. The hybrid molecule of claim 1, wherein said chemical entity comprises a radioactive moiety.

14. The hybrid molecule of claim 1, wherein said hybrid molecule is capable of affecting the rate of proliferation of cells bearing said high-affinity receptors.

15. A method for selectively binding a moiety to a cell bearing a high-affinity receptor for

a cyt kine, said method comprising exposing said cell to the hybrid molecule of claim 1, provided that said chemical entity comprises said moiety.

16. The method of claim 15, wherein said moiety comprises a label and said method is used for imaging said cell or said high affinity receptor.

17. The method of claim 15, wherein said moiety comprises a molecule complexed with iron and said method is used for separating cells having a relatively high number of said high-affinity receptors from other cells having a lower number of said high-affinity receptors.

18. The method of claim 15, wherein said hybrid molecule is capable of decreasing the rate of proliferation of cells to which it binds, and said method is used for therapeutic treatment of a condition comprising overproduction of cells having said high-affinity receptors.

19. A method for using the hybrid molecule of claim 1 for selectively isolating cells having said high-affinity receptors out of a population of cells some of which lack said high-affinity receptors, said method comprising,

immobilizing said hybrid molecule,

causing said population of cells to contact said immobilized hybrid molecule under conditions permitting binding of said high-affinity receptors to said immobilized hybrid molecule, and

separating unbound cells of said population of cells from cells bound via said high-affinity receptors to said immobilized hybrid molecule.

20. The method of claim 19, wherein said method is used to remove, from bodily fluids or tissues, cells having said high-affinity receptors.

21. The method of claim 19, said method additionally comprising the step of eluting from said immobilized hybrid molecule any cell which was bound to said immobilized hybrid molecule.

22. A method of making the hybrid molecule of claim 4, said method comprising

providing a recombinant DNA molecule comprising a DNA sequence encoding said polypeptide fused to a DNA sequence encoding said cytokine or said fragment or analog thereof,

introducing said recombinant DNA molecule into an appropriate expression system, and

expressing said recombinant DNA molecule.

23. A method of using the streptavidin-containing hybrid molecule of claim 9 for imaging said high-affinity receptors, or for imaging, in a population of cells, a cell bearing said high-affinity receptors, said method comprising

exposing said high-affinity receptors to an amount of said hybrid molecule sufficient substantially to maximize the difference between (1) the amount of said hybrid molecule which binds to said high-affinity receptors, and (2) the amount which binds to all other receptors on said population of cells;

exposing receptor-bound hybrid molecules to a labelled probe, said labelled probe being covalently linked to biotin; and

detecting said labelled probe which binds to said receptor-bound hybrid molecules.

24. The hybrid molecule of claim 4, wherein said polypeptide comprises an enzymatically-active fragment or analog of gelanin.

1 HisAlaProThr
1

TCTAGCTCTACCAAGAAAACCCAGCTGCAGCTCGAGCACCTGCTGCTGGATTTGCAGATG

SerSerSerThrLysLysThrGlnLeuGlnLeuGluHisLeuLeuLeuAspLeuGlnMET

5 10 15 20

ATCCTGAACGGTATCAACAATTACAAGAACCCGAAACTGACGCGTATGCTGACCTTCAAG

IleLeuAsnGlyIleAsnAsnTyrLysAsnProLysLeuThrArgMETLeuThrPheLys

25 30 35 40

TTCTACATGCCGAAGAAGGCCACCGAACTGAAACACCTGCAGTGTCTAGAAGAAGAACTG

PheTyrMETProLysLysAlaThrGluLeuLysHisLeuGlnCysLeuGluGluGluLeu

45 50 55 60

AAACCGCTGGAGGAAGTTCTGAACCTGGCTCAGTCTAAAAACTTCCACCTGCGGCCGCGT

LysProLeuGluGluValLeuAsnLeuAlaGlnSerLysAsnPheHisLeuArgProArg

65 70 75 80

GACCTGATCTCTAACATCAACGTAATCGTTCTGGAACTGAAGGGCTCTGAAACCACCTTC

AspLeuIleSerAsnIleAsnValIleValLeuGluLeuLysGlySerGluThrThrPhe

85 90 95 100

ATGTGTGAATACGCTGATGAGACCGCAACCATCGTAGAATTCCTGAACCGTTGGATCACC

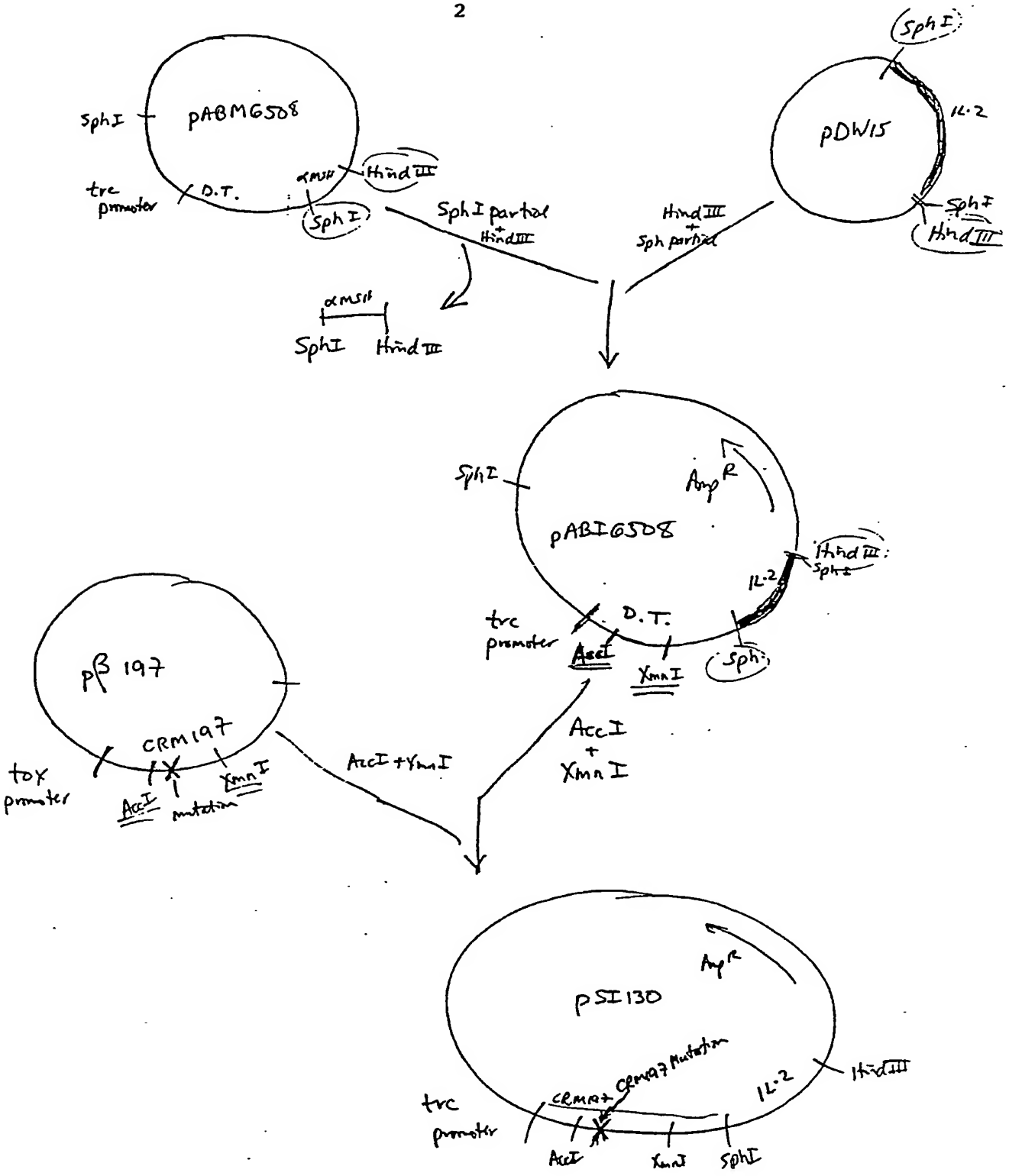
METCysGluTyrAlaAspGluThrAlaThrIleValGluPheLeuAsnArgTrpIleThr

105 110 115 120

TTCTGT CAGTCTATCATCTCTACCCTGACC

PheCysGlnSerIleIleSerThrLeuThr---

125 130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SphI
trc promoter
D.T.
αmsII
HindIII
SphI
SphI partial + HindIII
HindIII + Sph partial
pDW15
IL-2
SphI
HindIII
αmsII
SphI
HindIII
pABIG508
SphI
AmpR
HindIII
SphI
IL-2
trc promoter
D.T.
AccI
XmnI
SphI
pβ197
CRM197
tox promoter
AccI
mutation
XmnI
AccI+XmnI
AccI + XmnI
pSI130
AmpR
CRM+
CRM197 Mutation
IL-2
HindIII
trc promoter
AccI
XmnI
SphI

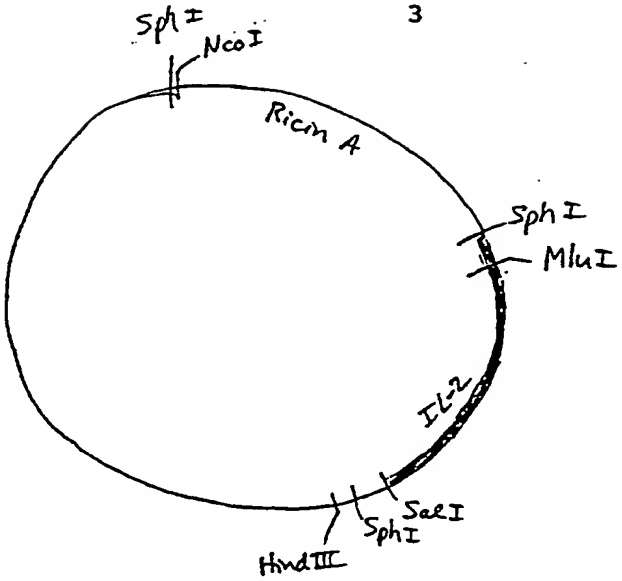
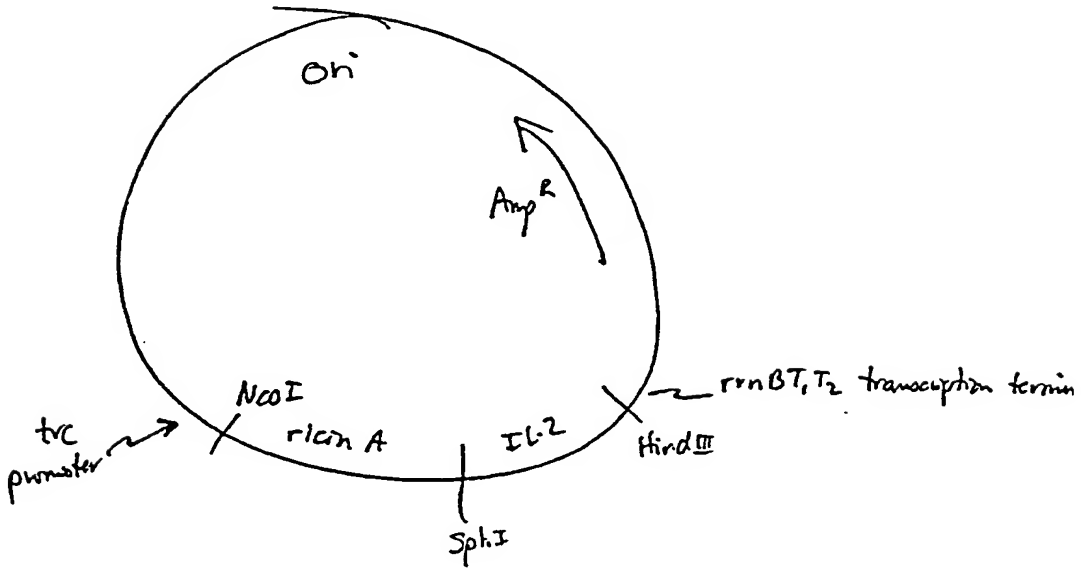

Sph I
Nco I
Ricin A
Sph I
Mlu I
IL-2
Sal I
Sph I
Hind III

FIG. 3(a)


ori
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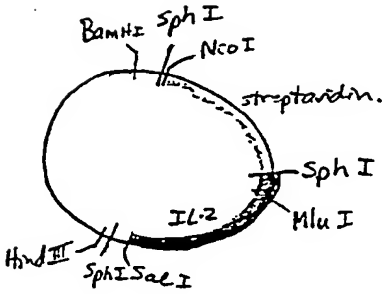
ricin A
IL-2
Hind III
rrnBT1T2 transcription termin
trc
promoter
Sph I

FIG. 3(b)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streptavidin.
Sph I
Mlu I
IL-2
HindIII
SphI
SalI

FIG. 4(a)

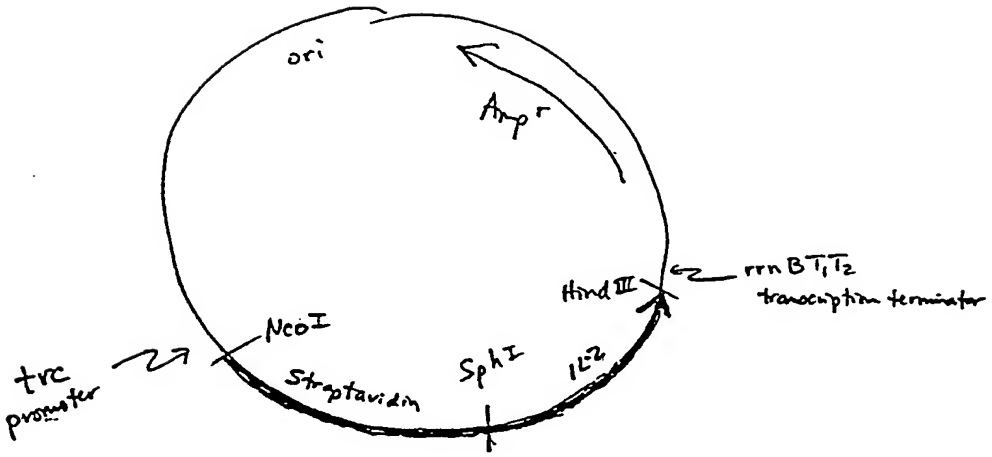
ori
Amp r
rrn B T1 T2
transcription terminator
Hind III
NcoI
trc
promoter
Streptavidin
SphI
IL-2

FIG. 4(b)

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/03828

I. CLASSIFICATI N OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): G01N 33/53; C12N 15/19; C07K 13/00
U.S. CL.: 435/7,172.3; 530/351

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/7,172.3,844; 530/351

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

CHEMICAL ABSTRACTS SERVICES ONLINE (FILE CA, 1967-1988; FILE BIOSIS PREVIEWS 1969-1988).

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with Indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	The Journal of Biological Chemistry, Volume 248, No. 11, issued 10 June 1973, T. Uchida et al., "Diphtheria Toxin and Related Proteins", pages 3838-3844, see abstract.	1-24
Y	Journal of Bacteriology, Volume 169, No. 11, issued November 1987, W. R. Bishai et al., "High-Level Expression of a Proteolytically Sensitive Diphtheria Toxin Fragment in Escherichia coli", pages 5140-5151, see abstract.	1-24
Y	The Journal of Biological Chemistry, Volume 263, No. 35, issued 15 December 1988, H. Lorber Boum-Galski et al., "Interleukin 2 (IL2) PE40 is Cytotoxic To Cells Displaying Either The p55 or p70 Subunit of The IL2 Receptor", pages 18650-18656, see abstract.	1-24

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
17 October 1990	10 DEC 1990
International Searching Authority [1]	**Signature of Authorized Officer** [20]
ISA/US	Laurie A. Scheiner

Form PCT/ISA/210 (second sheet) (May 1986)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Science, Volume 238, issued 23 October 1987, L. Greenfield et al., Mutations In Diphtheria Toxin Separate Binding From Entry And Amplify Immunotoxin Selectively", pages 536-539, see abstract.	1-24
Y	Nucleic Acids Research, Volume 16, No. 22, issued 1988, D. P. Williams et al., "Design, Synthesis and Expression of a Human Interleukin-2 Gene Incorporating The Codon Usage Bias Found in Highly Expressed Escherichia coli Genes", pages 10457-10467, see abstract.	1-24

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.